Original Article

The Relationship between Polymorphisms of Interleukin-4 Gene and Silicosis*

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Abstract

Objective To explore the relationship between polymorphisms of interleukin-4 (IL-4) gene (-33, +45, intron3, +429, +448) and the susceptibility of silicosis.

Methods A case-control study was carried out. 101 silicosis patients were selected as cases. As strictly matching, 121 of non silicosis workers were selected as the controls. The polymophisms of IL-4 (five locus) were detected by the method of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) techniques.

Results The GA genotype in the IL-4+429 locus and the CC genotype in the IL-4+448 locus were found. The frequencies of AA, GG and AG of IL-4+45 locus in the cases were 55.4%, 10.9%, and 33.7% and in the controls were 62.0%, 11.6%, and 26.4%. The differences between cases and controls were not significant. The frequencies of B1B1, B2B2, and B1B2 of intron3 VNTR locus in the cases were 73.3%, 1.0%, and 25.7% and in the controls were 68.6%, 1.7%, and 29.8%. The differences were not significant. The frequencies of TT, CC, and CT in -33 locus in the cases were 55.4%, 11.9%, and 32.7% and in the controls were 69.4%, 4.1%, and 26.4%. The differences were significant (*P*=0.034).

Conclusion The relationship between genetic polymorphism of IL-4-33 site and silicosis has been found and -33TT is a protective genotype for silicosis.

Key words: Silicosis; Interleukin-4; Polymorphism; Susceptibility

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INTRODUCTION

Silicosis is a fibrotic lung disease which is induced by the inhalation and deposition of silica dust. Cytokines participate in the development and progression of silicosis[1]. IL-4 is a potent cytokine which can promote fibrosis and enhance production of collagen both *in vitro* and *in vivo*[2]. Therefore, IL-4 may possibly promote the occurance of silicosis. The development of silicosis is related not only to environmental factors but also to the individual differences. The research on the relationship between polymorphisms of Interleukin-4 gene and susceptibility of silicosis has not been reported yet. The reseaches conducted recently were almost on the relationship between polymorphisms of IL-4 (-590, -33, intron3) and disease. Emi E. Nakayama found the C-to-T change at position -590 was completely associated with the C-to-T change at position -33[17]. Shweta Choudhry found that there was no relationship between the polymorphisms of IL-4 (+45, -33) and asthma[3], but an opposite study indicated that IL-4 -33 (C/T) in a conserved element (CNE-C) in the IL-4 gene, may influence susceptibility in combination with other variation in IL-4, or may merely be in LD with other

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variation in the gene that influences susceptibility to asthma and atopic phenotypes. The site of -33C/T in CNE-C was associated with asthma and atopy only when combined with other SNPs in the IL-4 gene. This SNP was previously associated with IgE levels in Japanese[4]. DONG found the polymorphism of the site had no relationship with the susceptibility of the disease of bronchiolitis caused by syncytial virus in the Wenzhou region. The NPSIL-4 and total serum lgE level were not correlated[5]. LIU suggested that polymorphism of IL-4 promoter site -33 was correlated with the susceptibility of asthma. It was an important candidate gene in children of the Han nationality in Hubei[6]. A different study by CHEN, SU, and others shown that purpura haemorrhagica and fibromyalgia were not related[7-8]. No relationship between the polymorphism of the IL-4+45 site and granular ophthalmia has been found by Angels Natividad[9]. CAO indicated that the VNTR was associated with COPD in the Han nationality in China[10]. LI found that childhood asthma was related to the polymorphism of allele B1 of IL-4 VNTR and the frequence was higher than that of the control group. It was possible that allele B1 contributed to the high expression of IL-4 in the asthma[11].

This study selected not only the two mutations sites of IL-4 (-33, intron3) , but also the three mutations sites which participated in the coding of amino acid, and they were IL-4 (+45, +429, +448). This study explored the relationship between Il-4 and silicosis in the level of gene in order to find more positive sites of polymorphisms. We are supposed to take the sites as the biological markers for screening workers' exposure to dust and take the susceptiblility of silicosis away from the dust environment, so that it can be a primary prevention measure for silicosis and have great significance in controlling the incidence of silicosis.

MATERIALS AND METHODS

Subjects

All the workers exposed to dust from the golden mine were examined by chest X-ray of dorsaventral base on the health check-up. The silicosis was diagnosed by the Pneumoconiosis Diagnosis Expert Panel, based on the Chinese National Diagnosis Criteria of Pneumoconiosis (GBZ70-2009). Among them, 101 silicosis patients were selected as cases and all of them were of the Han nationality. The cases were classified according to the working type and 5-year level exposure to dust. Workers without disease were selected as the controls, who were of the same nationality, with the same work type and level of exposure to dust with the cases. The control group included 121 persons. All people have signed a study informed consent. Questionnaires designed by ourselves were used and the data was collected by the face-to- face approach. A lot of imformation was collected, including general state of health, smoking status and past medical history. Management cards of workers exposed to dust were also used to verify and collect the imformation about the working place, workshop, age beginning to be exposed to dust, cumulative length of service exposure to dust, change in the work type, the time when diagnosised as pneumoconiosis, stage and whether there are complications or not.

Methods

Amplification DNA Peripheral venous blood was drawn from each subject and anticoagulated with 2% EDTA. The salt fractionation was used to extract DNA and then stored in -20 °C freezer. The PCR products were then resolved by electrophoresis in agarose gels, stained with ethidium bromide and photographed under ultraviolet light. The amplify-cation results were decided according to the location of PCR products after electrophoresis accompaning the reference of DNA marker location. The samples that were not amplified were amplified for a second time. The primers were synthesised and amplified according to the gene pool based on Pubmed, designing software of Premier Primer 5 and reports in literature. Primers used for IL-4 -33 were 5' –GTGCTGATTGG CCCCAAGTGAC-3' and 5'–TGGACTGCCACCAACCACCAGT-3'. PCR was performed in a 20 μL rection mix containing: 0.5 μL of dNTP, 2.0 μL of 10×PCR buffer, 2.0 μL of dye, 1.0 μL DNA templates, 1.0 μL of each of the primers, 0.2 μL of TaqDNA pclymerase, 12.3 μL of double distilled water. Cycling conditions of PCR were 2 m at 94 °C, then 35 cycles of 20 s at 94 °C, 30 s at 61.2 °C, and 30 s at 72 °C, and followed by an elongation step at 72 °C for 5 m. After that, the products were stored at 4 °C freezer. Amplification products had a 434 bp fragment; Primers used for IL-4+45 were 5' -TCTCACCTCCCAACTGCT-3' and 5-ACTTCCTACAAACCCTCA-3'. The cycling conditions of PCR were 2 min at 94 °C, 94 °C 20 s, 49.1°C 30 s, 72 °C 25 s, 35 cycles, 72 °C 5 min. Amplification product had a 303 bp fragment; Primers used for IL-4 VNTR were 5'-TAGGCTGAAAGGGGGAAAGC-3' and 5'

-TAGGCTGAAAGGGGGAAAGC-3'. Cycling conditions of PCR were 2 min at 94 °C, 94 °C 20 s, 60 °C 30 s, 72 °C 20 s, 35 cycles, 72 °C 5 min. Amplification products had 182 p or 252 bp fragments. Primes used for IL-4+429 were 5'-TCTCAGTATTTCTAGGCA-TGAAAACGT-3' and 5'-AGCAAAGATGTCTGTTAGATC-AACTC-3'. Cycling conditions of PCR were 2 min at 94 °C, 94 °C 20 s, 55.7 °C 30 s, 72 °C 20 s, 35 cycles, 72 °C 5 min. Amplification products had a 266 bp fragment; Primes used for IL-4+448 were 5'-TAACTCTGTCTCTTGCTCTCTCAT-3' and 5'-GTGGGACGGCTTCTTACCTTGGG-3'. Cycling condition of PCR were 2 min at 94 °C, 94 °C 20 s, 48.3 °C 30 s, 72 °C 15 s, 35 cycles, 72 °C 5 min. Amplification products had a 105 bp fragment.

Polymorphism Analysis BsmAI endonuclease (including 2 μL of 10×Buffer, 0.5 μL of BsmAI enzyme, 10 μL of PCR product, 17.5 μL of double distilled water) were for amplification products of IL-4 -33 for 10 h at 37 °C, and 8 μL sample were used for electro-phoresis. The CC genotype generated restriction fragments (of 192 bp and 242 bp), the CG (of 38 bp, 154 bp, 242 bp, and 242 bp), the GG of (38 bp, 154 bp, and 242 bp). NHEI endonuclease was for amplification products of IL-4 +45. The time was 10 h and the temperature was 37 °C; the GG genotype generated restriction fragments of 303 bp, the AG (of 37 bp, 266 bp, and 303 bp), the AA (of 37 bp and 266 bp). VNTR genotype can be decided by amplification products. B1B1 had a 182 bp fragment; B1B2 had two fragments of 182bp and 252bp; B2B2 had a 252 bp fragment. BcII endonuclease were for amplification products of IL-4 +429 for 8 h at 55 °C. The GG genotype generated restriction fragments of 266 bp, the GA (of 22 bp, 244 bp, and 266 bp), the AA (of 22 bp and 244 bp). BsuRI endonuclease were for amplifiction products of IL-4+448 for 10 h at 37 °C. The CC genotype generated restriction fragment of 105 bp, the CG (of 24 bp, 81 bp, and 105 bp), the GG (of 24 bp and 81 bp).

Statistical Analysis Excel was used for database and SPSS13.0 software was used for statistical analyses. Allele frequency=(number of homozygous of the gene ×2 + number of heterozygote of the gene)/(total number×2). Continuous variables were analyzed by Student's *t*-test and presented as means±SD. Pearson's χ^2 test was used to examine differences in characteristic variables and the distribution of genetic polymorphisms between cases and controls. The odds ratio (OR) and 95% confidence interval (95% CI) were for estimating the associations between the allele and the risk of silicosis. Values of $P<0.05$ were considered statistically significant.

RESULTS

In General

The average age was 59.1±8.9 years in the case group, ranging from 46 to 82 years; and average cumulative length of service exposure to dust was 28.0±5.1 years, ranging from 15 to 38 years. The average age was 57.3±8.1 years in the control group, where the controls were the same in sex and nationality as the cases. The ages of the controls ranged from 43 to 80 years, and average cumulative length of service exposure to dust was 28.1±5.2 years, varying from 14 to 39 years. Significant differences in age between the cases and the controls were not found ($P>0.05$) and significant differences in the cumulative length of service exposure to dust between the cases and the controls were also not found ($P>0.05$). The work types in the two groups matched with each other. The smoking rates in the two groups were 66.3% and 69.4% respectively and there were no significant differences in this regard ($P>0.05$).

The Comparison of Genotype and Allele Frequency in the 5 sites of IL-4

The allele frequency of IL-4-33T was less in the cases than in the controls; and the genotype frequency of CC was more than that in the controls and the differences were significant($P<0.05$). No significant difference was found based on genotype in IL-4+45 and VNRT sites between the cases and the controls. It showed that there was only GA genotype in IL-4+429 locus and there was no GG or AA genotype. Only CC genotype in IL-4+448 site was found, while CG and GG genotypes were not found in the site. The results were shown in Tables 1,2,3.

Table 1. The Genotypic Frequencies of IL-4 (-33, +45)

Groups	*n*	-33 site(%)			+45 site(%)		
		CC	CT	TT	AA	AG	GG
Cases	101	12 (11.9)	33 (32.7)	56 (55.4)	56 (55.4)	34 (33.7)	11 (10.9)
Controls	121	5 (4.1)	32 (26.4)	84 (69.4)	75 (62.0)	32 (26.4)	14 (11.6)
		χ^2=6.751, $P<0.05$			χ^2=1.386, $P>0.05$		

Table 2. The Genotypic Frequencies of IL-4 (VNTR)

Groups	*n*	VNTR site(%)			χ^2	*P*
		B1B1	B1B2	B2B2		
Cases	101	74(73.3)	26(25.7)	1(1.0)	0.580	>0.05
Controls	121	83(68.6)	36(29.8)	2(1.7)		

Table 3. The Allele Frequencies of IL-4 (-33, +45, and VNTR)

Groups	*n*	-33 site(%)		+45 site(%)		VNTR site(%)	
		C	T	A	G	B1	B2
Cases	101	57 (28.2)	145 (71.8)	146 (72.3)	56 (27.7)	174 (86.1)	28 (13.9)
Controls	121	42 (17.4)	200 (82.6)	182 (75.2)	60 (24.8)	202 (83.5)	40 (16.5)
OR	-	1.872		0.850		1.231	
95%CI	-	1.191-2.942		0.56-1.31		0.729-2.078	

DISCUSSIONS

More than two hundred million people are threatened by occupational diseases in China. Pneumoconiosis accounts for 80% in total occupational diseases and silicosis accounts for nearly 50% in pneumoconiosis. The cause of pneumoconiosis is clear and its incidence is related to both environmental and individual factors. Although exposure to dust is a start-up factor, only part of those exposed to dust get silicosis. Some others do not get pneumoconiosis all their lives. These facts suggest that individual factors also play a very important role in occurrence of pneumonoconiosis[12].

IL-4 has significant anti-inflammatory and fibrosis promotion functions. It has been found in the process of pathogenesis of allergic disease. IL-4 could attenuate acute inflammation. It also could increase the bronchial hyperresponsiveness[13]. IL-4, a type II cytokine, has been implicated in the pathogenesis of fibrosis by in vitro studies, which have shown that this cytokine could regulate fibroblastic function including chemotaxis, proliferation, collagen synthesis, and myofibroblastic differentiation[14].

Human IL-4 gene is located on chromosome 5, including four exons and three introns, about 10 kb, composed of 129 amino acids composition[15]. IL-4 is secreted by a variety of cells, such as: T cells, mast cell, antigen presenting cells and NK cells, etc. IL-4 can promote the secretion of TH2 type cytokines and has a decisive role in enhancing its effectiveness in the regulation of th1/th2 balance by inhibiting the secretion of TH1-type, but the secretion is influenced by the genetic information and other cytokines[16].

We concentrated to work on five polymorphisms: IL-4-33 as a significant site may play a role of biomarker for silicosis; IL-4 VNTR or +45 have no relationship with silicosis; we have not found the polymorphisms of IL-4+429 and +448.

IL-4-33 (C/T) presents in intron 1. It's the transcriptional start site. It is suggested by literature that it could enhance promoter activity and IL-4-33 (C/T) had relationship with allergic diseases. In the present study, we found that TT genotype of IL-4-33 might be a protective factor for the occurance of silicosis. In the other studies, the mutation of this site served as a risk factor or played a useless role, but it had protective function in silicosis. Therfore, it is specificity for silicosis. IL-4-33T accelerates disease progression in HIV-1 in-fection. It is also possible that IL-4-33T accelerates disease progression through suppression of cellular immunity, which plays an important role in controlling HIV-1 in infected individuals[17]. The SNP at position - 33 related to the transcription initiation site has also been found to enhance promoter activity of the gene and to be associated with atopic diseases. The -33 site had not relationship with malaria [18]. If more studies can prove our conclusion, the site -33 of IL-4 would be a prevention biomarker for silicosis.

IL-4+45 (A/G) is involved in encoding the amino acids, but the mutation of the site does not change the amino acid(Leu). All of related studies indicate that the mutation of IL-4+45 site could not change the function of IL-4 and has no relationship with silicosis and other diseases. VNTR is tandem repetitive sequence (TRS) of 70bp in the intron3 of IL-4. Recent studies indicate that the mutation of IL-4 VNTR may be a risk factor for disease of the respiratory system, but it does not have evident effects on silicosis. We learned that the two sites of IL-4 (+429G/A, +448C/G) present in exon 1 by Pubmed gene bank were involved in the coding of 53 and 59 amino acids respectively; the mutations of them can cause the change of corresponding amino acid, Val into Ile and Ala into Gly respectively. There has been no reports about these two sites. We found that all the genotypes in site IL-4+429 were all heterozygote in the workers exposured to dust and all the genotypes in IL-4+448 site were all wild type. Such results may be closely related with the Asian race, which need to be studied further. Most of the results are negative but ture and credible. This indicates that the function of the four sites have no

differences in terms of their association with silicosis.

Individual SNPs are considered to make a small contribution to disease risks in most of the cases. Their potential effects may escape detection when examined separately. Previous studies have found that many sites of polymorphisms were related to silicosis including IL-1α-889, IL-6-634, FN-Msp I, FN-Hae III b, PDGF-D (3166), etc. Therefore, combined analyses of a series of SNPs may be more suitable than separate calculations to identify an existing relationship with silicosis[19]. We expect to find the pathopoiesis gene of silicosis.

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